2

The Effect of Thermal Acclimation on Brain Cholinesterase Activity of the Killifish, *Fundulus heteroclitus*[1]

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(Text-figures 1-5)

Introduction

The purpose of this study was to investigate some of the biochemical factors involved in the functional failure of the central nervous system in response to thermal stress in poikilothermic animals. The effect of thermal stress on the enzymatic activity and properties of brain cholinesterase (ChE) from the killifish, *Fundulus heteroclitus,* after a period of acclimation to high and low temperatures, has been investigated and an attempt has been made to correlate some of these findings with physiological and electrophysiological data that have been reported previously.

The studies on temperature acclimation in fish reviewed by Brett (1956) and Fisher (1958) indicate that acclimation to thermal stress occurs on the organism, organ, tissue, cellular and biochemical levels. In addition, these reviewers and others (Fry, 1947; and Roots & Prosser, 1962) suggest that the ultimate cause of heat and cold death in fish is intimately associated with a failure of the central nervous system. Since the upper and lower lethal temperature limits for fish vary directly with the acclimation temperature (Brett, 1956), it is clear that some modification occurs in the nervous system in response to thermal stress, which alters its physiological state.

Fries (1952) has observed chill coma in fish and describes a syndrome of numbness, convulsion and pectoral paralysis just preceding death. This entire syndrome, including the striking condition in which the pectoral fins are immobile and stand at a 90° angle to the body, can be duplicated in fish by the action of various anticholinesterases (Weiss, 1961), which suggests that cold death in fish may be due to the physiological limits set by the enzyme ChE of nerve tissue.

The enzymatic activity and some of the properties of fish brain cholinesterases have been investigated (Nachmansohn *et al.,* 1941; Lindeman, 1945; Augustinsson, 1948, 1949; Augustinsson & Fange, 1950; Brightman & Albers, 1959; Baslow & Nigrelli, 1961; Weiss, 1958, 1959, 1961, and Brik & Yakovlev, 1962). The work described in this paper concerns the effect of altered ambient temperature on the activity and some of the properties of fish brain ChE initially, during and after thermal acclimation to high and low temperatures.

Materials and Methods

The animals used in this investigation were essentially marine or brackish water fishes, inhabiting a wide range of temperature zones. Unless otherwise indicated, the northern fishes were adapted to 15° C and the southern fishes to 25° C. All were in prime condition at the time of sacrifice. Fish used for adaptation experiments were maintained on a diet of fish, chopped clams and frozen brine shrimp. The killifish, *Fundulus heteroclitus,* was used as an experimental animal for acclimation studies because of its hardiness, ability to tolerate large temperature variations and availability in large homogeneous populations. The temperatures to which this species was exposed were similar to their normal summer and winter thermal range.

North Atlantic fishes:

Common killifish, *Fundulus heteroclitus;*

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northern blackfish, *Tautoga onitis;* common goldfish, *Carassius auratus* (acclimated to 22° C); northern stargazer, *Astroscopus guttatus.*

South Atlantic and South Pacific fishes:

Nassau grouper, *Epinephelus striatus;* Holbrook's porgy, *Diplodus holbrookii;* spot snapper, *Lutianus synagris;* fantail filefish, *Monocanthus spilosoma;* tomato clownfish, *Amphiprion frenatus;* anemone clownfish, *Amphiprion percula.*

Fishes used for acclimation studies were kept in a ten-gallon running sea water system, fed from a ten-gallon reservoir in which the water temperature could be maintained at any temperature between 12 and 35° C, plus or minus one-half degree. The fresh sea water, which came in from a well point at 12° C, was aerated and adjusted to the proper temperature with two thermostatically controlled 250-watt heaters prior to flowing into the experimental tank.

The assay technique used was the colorometric method described by Hestrin (1949), which utilizes a reaction between hydroxylamine and unhydrolyzed acetylcholine. This is the method used by Weiss (1958, 1959) for the determination of fish brain ChE after treatment with various anti-cholinesterases. Fish brains were obtained by removing the top of the skull and cutting the brain loose at the optic nerves and the base of the medulla, after the fish was incapacitated by severing the spinal cord at the level of the pectoral fins. The brains were then washed in cold barbital buffer solution (Lundin, 1959) to remove blood clots, weighed and then homogenized in a ground glass homogenizer in a small amount of buffer (pH 8.1). The resulting brain brei was diluted with additional buffer to 1-4 milligrams of brain tissue per milliliter of solution.

For analysis, one-half milliliter of diluted brain brei was pipetted into a test tube, preincubated for 5 minutes at the assay temperature, and then incubated with one-half milliliter of 0.008 M acetylcholine chloride (Augustinsson, 1949). The standard assay time was 20 minutes at 26° C, plus or minus 0.5° C. Residual substrate was determined by the method of Hestrin (1949) and the enzymatic activity of the brain tissue is reported in terms of milligrams of substrate (acetylcholine chloride) hydrolyzed by the enzyme present in 100 milligrams of tissue per hour. This value is notated Q_{ChE}.

Observations and Results

A. Effects of temperature variation on brain ChE activity *in vitro.*

1. Determination of Q_{10} of ChE. The Q_{10} or factor for increase in enzymatic activity for a 10° C rise in temperature of brain ChE from various species of fish was determined by incubation of enzyme and substrate at different temperatures from 0 to 40° C. It was found that the Q_{10} for the enzyme from each species of fish was fairly constant over a wide temperature range (10-35° C), and thus could be used to determine the specific enzyme activity for any temperature within this range once a standard determination had been made. Below 10° C the Q_{10} of ChE from several fish species increased greatly, thereby causing a significant loss of enzyme activity within a few degrees. It is interesting to note that this occurred in preparations from the filefish and snapper, two "southern" fishes. In a number of "northern" fishes whose brain ChE was analyzed at low temperatures, including the goldfish, blackfish and killifish, the Q_{10} for this enzyme remained fairly constant down to 2° C, at which point the Q_{10} increased and the enzymatic activity decreased sharply as it did in "southern" fishes. The Q_{10} values for brain ChE from killifish acclimated to 12 and 30° C were similar. The possibility that

Table I. Analysis of Various Fish Species for Brain ChE Activity and Enzymatic Q_{10} Values.

Species	Brain weight (mgs)	Total body weight (gms)	Q_{ChE} at 26°C	Q_{10} 15-25°C
Common goldfish	70.3	6.3	41	1.40
Northern blackfish	153.0	60.0	57	1.19
Common killifish	29.7	5.6	48	1.30
Northern stargazer	41.2	58.5	46	1.25
Holbrook's porgy	407.4	221.0	57	1.17
Nassau grouper	895.2	6940.0	28	1.25
Tomato clownfish	32.1	3.8	36	1.40
Anemone clownfish	23.6	2.3	53	1.45
Spot snapper	904.0	937.6	41	1.15
Fantail filefish	113.0	21.0	99	1.02

TABLE II. ANALYSIS OF THE DENATURATION OF BRAIN ChE FROM VARIOUS FISH SPECIES AT ELEVATED TEMPERATURES *in vitro*.

Species	Incubation temperature (°C)	Incubation time (min.)	Loss of ChE activity (%)
NORTH ATLANTIC FISHES			
Common goldfish	26	30	0
	40	30	6
	40	60	12
Northern blackfish	26	120	0
	35	120	5
	40	30	41
Common killifish	26	60	0
	34	60	22
	40	15	50
	40	30	78
	40	60	100
SOUTH ATLANTIC AND SOUTH PACIFIC FISHES			
Tomato clownfish	26	60	0
	35	60	11
	40	30	9
	40	60	21
Anemone clownfish	35	60	7
	40	30	13
	40	60	21
Spot snapper	35	60	0
	40	60	11
Fantail filefish	35	60	0
	40	90	6

there are differences in the properties of ChE from warm and cold living fishes is indicated.

In Table I representative values for enzymatic activity (Q_{ChE}) measured at 26° C and Q_{10} for brain ChE from several species of fish are given. Q_{10} values range from 1.02 to 1.45 and are derived from measurements made at 15 and 25° C.

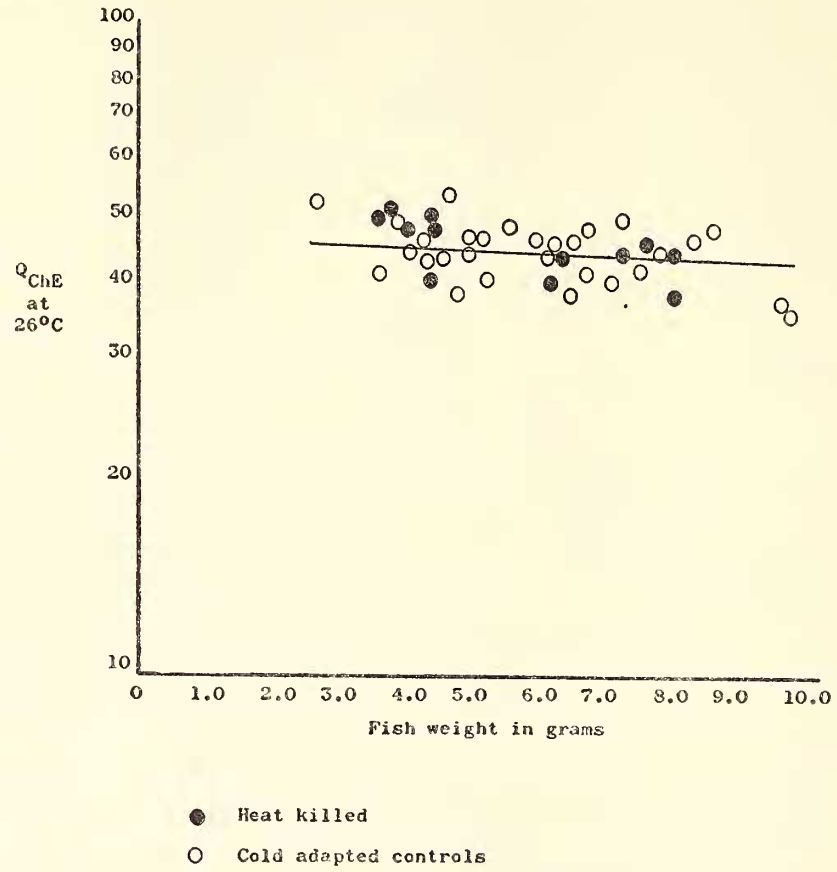
2. Enzyme denaturation at elevated temperatures. At temperatures above 30° C it was found that brain ChE from various sources became rapidly inactivated *in vitro*. In order to determine the degree of denaturation quantitatively, the enzyme preparation was pre-incubated at elevated temperatures for various periods of time, cooled, and then assayed at 26° C with the substrate acetylcholine for residual enzymatic activity. These results are shown in Table II.

Although the number of species involved is small, it is evident that the warm water species exhibit a more heat-stable enzyme than the cold water species, which again indicates basic differences in the properties of brain ChE from cold and warm water forms.

B. Effects of temperature variation on brain ChE *in vivo*.

1. Effect of heat death on brain ChE activity levels of the killifish *Fundulus heteroclitus*. In order to determine if the ChE degradation found to occur at elevated temperatures *in vitro* also occurred *in vivo*, a number of animals were heat killed and their brains analyzed for residual enzymatic activity. The fish were obtained from cold sea water (13° C) and placed in an aerated three-gallon glass aquarium. The temperature was raised to 30° C within 30 minutes, and to 35° C in another 20 minutes. The fish were killed by raising the water temperature to 40° C within the next 16 minutes. The endpoint was reached when the animals floated at the surface and twitched occasionally, but had no control over their swimming movements. After cutting the spinal cord the fish were plunged into an ice water bath and prepared for assay along with a control group of fish. The results of this experiment are seen in Text-fig. 1 and it will be noted that there are no differences between heat killed and control animals.

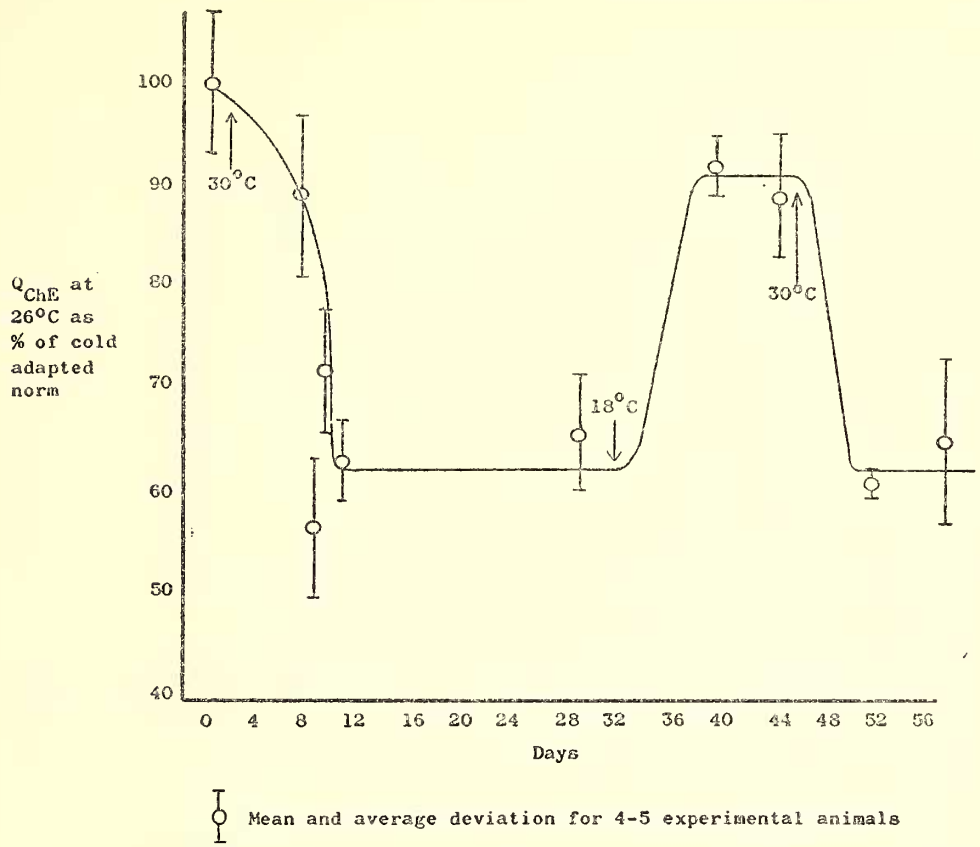
2. Effect of temperature stress on brain ChE activity levels of the killifish, *Fundulus heteroclitus*. To determine the effect of temperature



TEXT-FIG. 1. Brain ChE activity of heat-killed *Fundulus heteroclitus*.

stress and acclimation on the activity of ChE in the central nervous system of the killifish *in vivo*, a large group of animals acclimated to 12° C were placed in a running sea water system capable of maintaining any temperature between 12 and 35° C, plus or minus one-half degree. These fish were then stressed over a period of eight hours by slowly increasing the ambient water temperature to 30° C. Brain samples from 4-5 experimental animals were analyzed for ChE activity on days 7, 8, 9, 10 and 29. After 32 days at this temperature, the population was again subjected to thermal stress by reducing the ambient temperature to 18° C. Determinations of brain ChE activity were made after 7 and 12 days at this temperature. After 13 days at 18° C this group of fish was once more stressed with a temperature of 30° C and samples were taken after 7 and 13 days for analysis of brain ChE activity. The results presented in Text-fig. 2 show that the brain ChE activity of the killifish varies inversely with the ambient temperature, and is approximately 40% lower after acclimation to 30° C than when acclimated to 18° C when the enzymatic activity is measured at 26° C. Non-stressed cold-acclimated killifish were used as controls when determinations of brain ChE activity of the experimental population were made.

A second group of cold-acclimated killifish was stressed with a temperature of 30° C and the changes occurring in brain ChE activity were followed daily, in addition to changes in the opercular rate. The results of this experiment are shown in Text-fig. 3. The reduction in brain ChE activity begins immediately in response to heat stress and is a time-dependent function, being completed in about six days. Opercular rate is also affected immediately upon warming and reduction to the prewarming level is also a time-dependent function which coincides with the reduction in ChE activity. After 7 days, this population was stressed with a temperature of 13° C and it was observed that the animals required about 5 days at the lower temperature before any elevation in ChE activity was evident. Thus, it appears that acclimation on the biochemical level occurs much more rapidly to



TEXT-FIG. 2. Brain ChE activity levels in response to thermal stress in *Fundulus heteroclitus*.

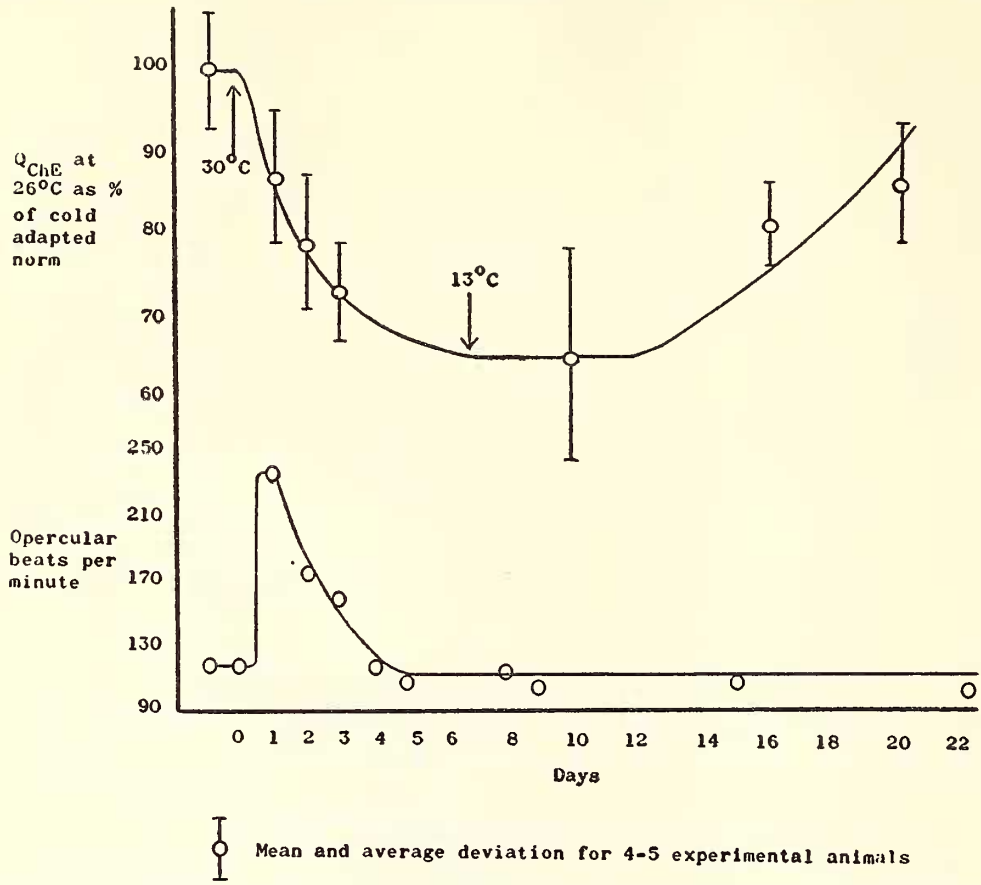
warm than to cold stress. It will also be seen in Text-fig. 4, when enzymatic activity is plotted as a function of the ambient temperature, that the effect of acclimation is to reestablish a specific enzyme activity of approximately Q_{ChE}=31 at each new temperature. The specific brain ChE activity of animals adapted to 13° C, when measured at that temperature, is approximately Q_{ChE}=31. When stressed with an ambient temperature of 30° C this value goes up to about 50. After a period of acclimation the ChE activity level drops again to about 31 when measured at the new ambient temperature. When the warm adapted animals are cooled to 13° C once again, the brain ChE activity drops to about Q_{ChE}=20 initially, but rises to approximately 31 after a period of acclimation to the lowered ambient temperature.

C. Seasonal variation of brain ChE activity of *Fundulus heteroclitus* when maintained at a constant temperature.

When the values for brain ChE of cold water control animals, measured at 26° C, are plotted by month against the October mean value (100%), it is apparent that a rise in brain ChE activity occurs. This rise is almost inperceptible at first, but at the end of January and beginning of February the ChE activity level rises rapidly and reaches a value of more than 130% of the October mean (Text-fig. 5). This variation occurred in a population of fish being maintained in a cold sea water reservoir whose temperature did not vary more than 2° C in five months. Since the population of fish as determined by weight and brain-body proportions did not change during this period, the rise in ChE activity could not be due to selection of smaller fish with higher brain Q_{ChE} activity levels.

Discussion

The results obtained in this investigation (Table I) for brain ChE activity in various teleost fishes are similar to those obtained by Augustinsson (1948) and show that large species differences exist. Even within the limited number of species examined, a three-fold variation in brain ChE levels was found, ranging in enzymatic activity levels from Q_{ChE} 28 to 99. When



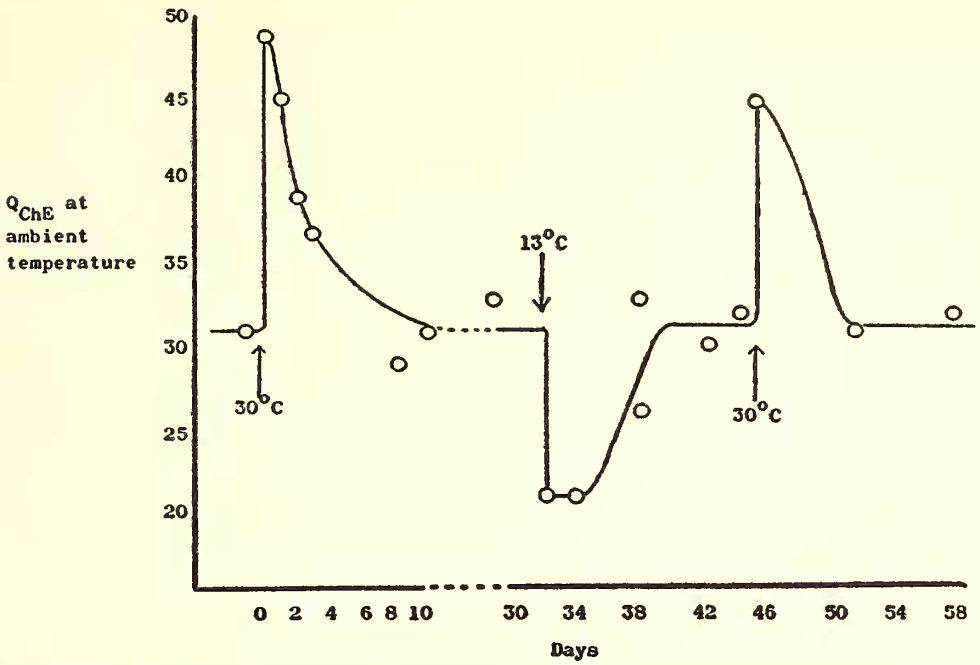
TEXT-FIG. 3. Brain ChE activity levels and opercular rate in response to thermal stress in *Fundulus heteroclitus*.

large populations of a single species are analyzed, however, individual variations are relatively small, usually between 5-10% of the mean ChE level of the species, providing that the body size and thermal history of the sampled population are similar (Text-fig. 1). This has been found for the killifish during this investigation, and has also been reported for the bluegill sunfish, *Lepomis macrochirus;* largemouth bass, *Micropterus salmoides*; golden shiner, *Notemigonus crysoleucas* and common goldfish, *Carassius auratus* (Weiss, 1959). Values for brain ChE activity have been reported for other fish species and the Q_{ChE} activity levels have been found to vary in teleosts from 0.5 to 60 (Nachmansohn *et al.,* 1941; Lindeman, 1945; Augustinsson, 1948; Augustinsson & Fange, 1950; Weiss, 1959; and Baslow & Nigrelli, 1961).

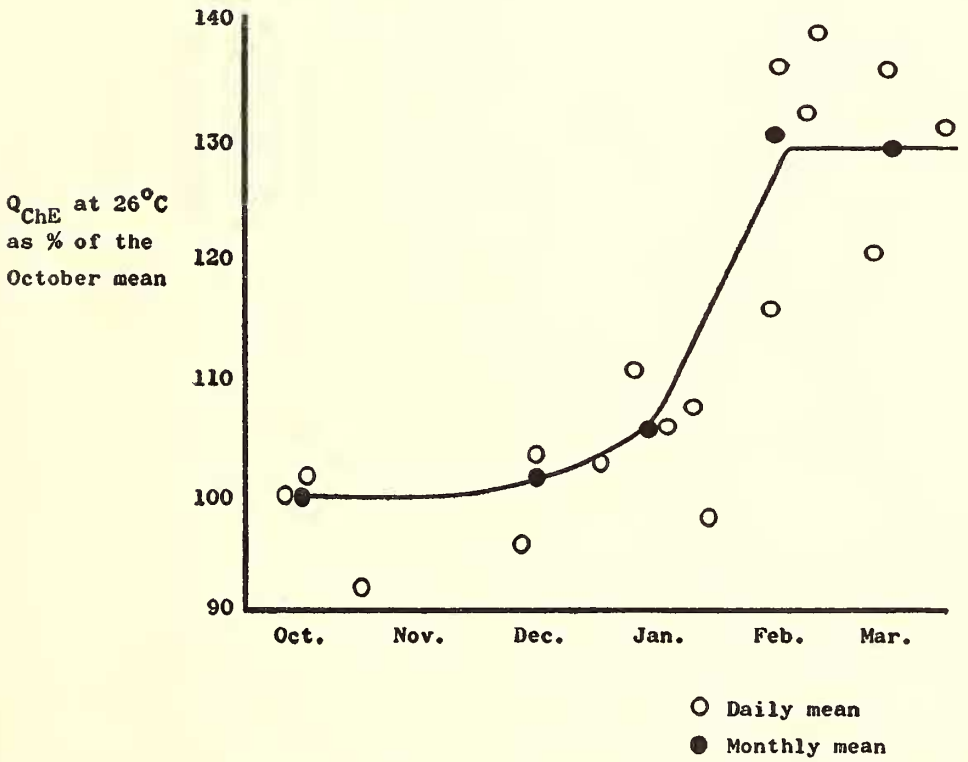
It has also been noted (Text-fig. 1) that as the size of the brain increases in *Fundulus heteroclitus* the ChE activity per unit weight of brain tissue decreases. This has been found to be true for the rat (Roderick, 1960) and also sunfish, largemouth bass, golden shiner, and common goldfish by Weiss (1958, 1959, 1961).

Determinations made over a wide temperature range *in vitro* show that the Q_{10} of brain ChE for several species of fish varies between 1.02 and 1.45 (Table I.) These values are similar to those described by Chadwick (1957) for cholinesterase from various sources but somewhat less than the value of 2 derived for the cholinesterase of the intestine of the catfish, *Ameiurus nebulosus,* by Laurent (1952).

Determinations of residual brain ChE activity after exposure to elevated temperatures have shown that ChE from various fish species is denatured rapidly above 30° C *in vitro* (Table II). This has also been demonstrated for brain oxidative enzymes of the goldfish (Freeman, 1950) and for the enzymes associated with oxidative metabolism of the brain of the largemouth bass by Fuhrman *et al.* (1944). The temperature of inactivation of those enzymes associated with oxidative metabolism *in vitro* corresponds very well, in many cases, with the known upper lethal



TEXT-FIG. 4. Enzymatic activity as a function of ambient temperature in *Fundulus heteroclitus.*



TEXT-FIG. 5. Seasonal variation in brain ChE activity of *Fundulus heteroclitus* when maintained at a constant temperature of 13° C.

temperatures for the respective fish species. In these cases, enzyme inactivation has been suggested as a possible cause of heat death due to exposure to high temperatures. In order to determine whether the brain ChE *in vitro* instability to elevated temperature also occurred *in vivo*, and to see if this instability could be responsible for functional failure of the central nervous system, "heat death" experiments were performed as described previously. The experimental animal *Fundulus heteroclitus*, which exhibited a very heat labile brain ChE *in vitro* (Table II), was exposed to environmental temperatures between 35 and 40° C for more than 20 minutes, or until heat death occurred. *In vitro*, this time and temperature exposure should have resulted in approximately a 30% reduction in brain ChE activity. The results show, however, that no enzyme denaturation had occurred at the time of heat death due to exposure to elevated temperature (Text-fig. 1). It appears, then, that the thermal instability of brain ChE observed *in vitro* does not occur *in vivo* and therefore cannot contribute to functional failure of the central nervous system at elevated temperatures.

It has been noted that the activity of many enzymes in poikilotherms adapts inversely to the ambient temperature when measured at a constant temperature (Precht, 1951; Christophersen & Precht, 1952; Stangenberg, 1955; Kono & Nakagami, 1957; and Kanungo & Prosser, 1959). This has been found to be true also for brain ChE of the killifish. The variation in enzymes and enzyme systems due to acclimation to temperatures approximately 15° C apart is of a large magnitude. In this investigation differences of 40% have been found for brain ChE levels in *Fundulus heteroclitus*. Kanungo & Prosser (1959) find a 43% increase in oxygen consumption of liver homogenates of cold-adapted goldfish, *Carassius auratus*, and Precht (1951) finds a 50% decrease in succinodehydrogenase activity in the muscle of the fresh water eel, *Anguilla vulgaris*, adapted to warm temperatures.

Acclimation to thermal stress in poikilotherms can be measured by following the response of any of a number of physiological processes, and Precht (1959) has shown that movements of the gill covers of the swordtail, *Xiphophorus helleri*, and the guppy, *Lebistes reticulatus*, exhibit a significant adaptation to heat and cold. Freeman (1950) has shown that opercular rhythm, oxygen consumption and brain metabolism adapt together in response to thermal stress in the goldfish. In this investigation the changes in response to thermal stress were determined daily for opercular rate and brain ChE activity of the killifish. The normal opercular rate of approximately 100 beats per minute found in cold adapted fish was abruptly raised to over 230 beats per minute as a result of thermal shock caused by increasing the environmental temperature from 12 to 30° C. The opercular rate at the elevated temperature, over a period of five days, gradually returned to the cold-adapted level of 100 beats per minute, and coincided with the daily observed reduction in ChE activity (Text-fig. 3).

In response to heat stress, about 7 days are required to reach a stable lowered brain ChE activity level in *Fundulus heteroclitus*. Wells (1935) has shown that heat adaptation in the Pacific killifish, *Fundulus parvipinnis*, requires at least eight days for completion. In response to cold stress, a "lag" period of about four days occurs, followed by an increase in ChE activity with the development of a new stabilized enzyme level in about eleven days. Thus, from the standpoint of time, it is observed that it is more difficult to adapt biochemically to a decrease than to an increase in ambient temperature (Text-figs. 2, 3). Experimentally, it has also been found that it is more difficult to adapt a fish to a drop than to an increase in environmental temperature (Brett, 1956).

When the activity of fish brain ChE is plotted at the temperature of acclimation it becomes clear that a homeostatic mechanism exists in the central nervous system of *Fundulus heteroclitus* which regulates the level of ChE activity. The initial effect of heat stress on this enzyme system is to increase the specific activity of the enzyme ChE. By stressing a fish living at 12° C with a temperature of 30° C, the ChE activity will have a Q_{ChE} value of over 50, which is almost double the enzymatic activity present at the lower temperature. The effect of cold stress is opposite to that of heat stress, with the specific activity level of brain ChE falling below $Q_{ChE}=31$. As seen in Text-fig. 4 the response of the central nervous system of the killifish to thermal stress is to re-establish the previous enzyme activity level at the new ambient temperature. This process occurs within and coincides with the observed periods of acclimation to temperature variations. In the killifish the activity level of brain ChE that is maintained at each ambient temperature is approximately $Q_{ChE}=31$.

The seasonal increase in brain ChE levels in the killifish under conditions of constant environmental temperature has been described (Text-fig. 5). Considering the possible survival value of this enzyme in response to thermal

shock, it is interesting that Hoar (1955) finds that the high and low lethal temperature limits of goldfish exhibit a seasonal cycle even under conditions of constant temperature. Wells (1935) found a seasonal variation in oxygen consumption of the Pacific killifish when kept at a constant temperature. Oxygen consumption rose in late January and early February to approximately 130% of the August through January mean and this rise in oxygen consumption occurred during that period of time when the lowest yearly water temperatures were recorded under natural conditions. The seasonal increase in brain ChE found in this investigation for *Fundulus heteroclitus* also occurred at the time of minimal recorded water temperatures in the fishes' natural environment. This fact, and the parallel increase in function (130%) occurring during the same four-week interval as that of the Pacific killifish, suggests that seasonal variations in physiology and biochemistry occur widely and may be important to poikilotherms that occupy environmental niches that are characterized by large seasonal variations in temperature.

Inactivation of brain and peripheral nerve ChE with specific anticholinesterases results in autointoxication due to the presence of unhydrolyzed acetylcholine at the synaptic junction. Under conditions of constant temperature, the residual quantities of brain ChE activity compatible with survival in fish, using anticholinesterase drugs, have been found to range between 20 and 60% of the normal level (Weiss, 1958, 1959, 1961). Nachmansohn & Feld (1947) have found an average value of 25% for a number of animals ranging between 10 and 50% of the normal activity level for this enzyme. The results of this investigation have indicated that the specific activity of brain ChE at any acclimation temperature is a constant as a result of biochemical adaptation. Thus, it is possible to calculate the ChE activity at the upper and lower lethal temperature if the Q_{10} and Q_{ChE} are known for any acclimation temperature. For the goldfish these values are 1.40 and 41 respectively (Table I). Fry *et al.* (1942) has found that goldfish acclimated to 20, 30 and 40° C have low lethal temperatures of 1, 9 and 17° C respectively. The calculated residual brain ChE activity at these temperatures are 52, 50 and 47% of the acclimated enzyme activity. The cold-blocking temperature of a simple tail reflex in goldfish acclimated to 25 and 35° C has been reported to be 5 and 10° C respectively (Roots & Prosser, 1962). In this case also, calculation of the residual enzyme activity indicates that this reflex activity stops when approximately 50% of the ChE activity has been lost.

The similarity in range of values for residual brain ChE activity due to both thermal and drug inactivation suggests that the primary cause of cold death and nerve block in this fish is due to failure of the central nervous system by physiological limitations set by experimentally induced or seasonal variation in ChE activity. The observed characteristics of cold death syndrome, such as numbness, muscular twitching, convulsion and paralysis (Fries, 1952; Roots & Prosser, 1962), are all indicative of physiological lack of this enzyme.

Summary

1. In response to experimentally-induced thermal stress in the killifish, *Fundulus heteroclitus*, changes in brain cholinesterase (ChE) activity levels were observed. These alterations in enzymatic activity vary inversely with the temperature of acclimation.

2. The observed changes in brain ChE activity in the killifish occur in such a way that a specific activity level of $Q_{ChE}=31$ (milligrams of substrate hydrolyzed per hour by the enzyme present in 100 milligrams of tissue) is maintained regardless of the ambient temperature. This is achieved after a period of acclimation indicating that a homeostatic regulatory mechanism exists in brain tissue governing the activity of this enzyme.

3. Differences in *in vitro* stability of brain ChE in various fish species were found in response to elevated temperatures. Warm water species seem to have a more heat-stable enzyme than cold water forms.

4. Exposure to high temperature sufficient to cause death in the killifish does not result in denaturation of brain ChE, indicating that the lability of this enzyme *in vitro* does not occur *in vivo* and that the inactivation of this enzyme cannot be considered to contribute to heat death.

5. Cold death in fishes may be due to physiological limits set by the enzyme ChE of nerve tissue. Thermal inactivation of this enzyme may cause autointoxication due to the accumulation of unhydrolyzed acetylcholine at the synaptic junction.

6. The Q_{10} or factor for the increase in enzymatic activity for a 10° C rise in temperature *in vitro* for brain ChE from various fish species is apparently a species characteristic. Q_{10} values range from 1.02 to 1.45. The enzyme from warm water fish seems to be inactivated more easily by low temperature than brain ChE from cold water species.

7. A seasonal variation in brain ChE has been observed in the killifish under conditions of constant temperature. A 30% rise in enzyme activity occurs shortly after the lowest yearly water temperatures have been recorded in the natural habitat of this fish.

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